Lactobacillus reuteri DSM 17938 alleviates galactosamine-induced liver failure in rats

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ARTICLEINFO

Keywords:
Lactobacillus reuteri DSM 17938
Liver failure
Gut microbiota
Metabolome
Transcriptome

ABSTRACT

Liver failure is a serious hepatic dysfunction with high mortality. This work aimed to investigate the effect of a famous probiotic and drug, *Lactobacillus reuteri* DSM 17938, on liver failure in rats. Sprague-Dawley rats were gavaged with 3×10^9 CFU of DSM 17938 for 7 days. D-galactosamine was intraperitoneally injected to induce acute liver failure on the eighth day. Samples were collected to determine the liver function, serum cytokines levels, terminal ileum and liver histology, gut microbiota, metabolome and transcriptome. Our results showed that pretreatment with DSM 17938 not only reduced the elevation in serum alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase, IL-1α, IL-2, IL-18, M-CSF, and MIP-3α levels but also alleviated histological abnormalities of both the terminal ileum and liver induced by D-galactosamine. Additionally, DSM 17938 reduced D-galactosamine-induced enrichment of some taxa of gut Actinobacteria or Firmicutes, including abundant pathogens such as *Actinomycetales*, *Coriobacteriaceae*, *Staphylococcaceae* and *Enterococcaceae*. Furthermore, DSM 17938 reduced the D-galactosamine-induced increase in not only fecal metabolites such as trisaminol and lithocholic acid but also the transcription of liver inflammatory genes, such as *Ccl2*, *Ccl7*, *Ccl11*, *Ccl12*, *Il6* *Il11*, *Il20rb*, *Mmp3* and *Mmp10*. Downregulation of retinol metabolism and PPAR signaling pathway as well as upregulation of viral protein interaction with cytokine and cytokine receptor and central carbon metabolism in cancer signaling pathway were involved in the mechanism of *L. reuteri* DSM 17938 alleviating liver failure. Our findings suggested that DSM 17938 is a potential probiotic for the prevention or treatment of liver failure.

1. Introduction

Liver failure is a life-threatening hepatic dysfunction or decompensation usually caused by direct or indirect injury to liver cells by viruses, drugs or cytokines [1]. The liver and gut are closely linked with several vital life activities such as shuttling of immune cells and cytokines, metabolism, and the enterohepatic circulation of bile acids. Large and complex microbial communities live in the human gut and play important roles in growth, development, immunity, metabolism and diseases [2]. Various acute and chronic liver diseases affect the gut microbiota composition while some gut microbiota alterations have vital effects on the occurrence and development of various liver diseases, particularly liver failure [3–5].

Probiotics, especially *Lactobacillus*, can inhibit the growth of pathogens and improve the immunology and metabolic functions of the host. Strains of *Lactobacillus reuteri* are common inhabitants of the mammalian gut and exhibit health-promoting properties [6]. L. *reuteri* DSM 17938 was derived from ATCC 55730 by removal of plasmids carrying tetracycline- and phthalamide-resistant genes [7,8]. *L. reuteri* DSM 17938 has been extensively used in adults and infants as a drug or probiotics [9–11]. *L. reuteri* DSM 17938 can reportedly prevent or reduce diarrhea, infantile colic, constipation, and gastrointestinal and respiratory infections [12–17].

In this work, the effect of *L. reuteri* DSM 17938 on acute liver injury was investigated using a D-galactosamine-induced rat liver failure model. Then, the mechanism involved in this process was further explored based on the integration of cytokine array, gut microbiota, metabolome and transcriptome data.

2. Materials and methods

2.1. Strains and culture conditions

L. reuteri DSM 17938 was isolated and identified from a product named Protectis baby (Biogaia AB, Sweden). After anaerobic incubation in MRS broth (Thermo Fisher, Shanghai, China) at 37 °C for 18 h, the culture of *L. reuteri* DSM 17938 was centrifuged at 8000 ×g for 3 min. Then, cells of *L. reuteri* DSM 17938 were washed three times with sterile normal saline and resuspended to a concentration of 3×10^9 colony- forming units (CFU)/mL for further use. *2.2. Experimental design and sample collection*

Twenty-four male specific pathogen-free (SPF) Sprague-Dawley rats (Shanghai SLAC Laboratory Animal Co., Ltd., China) weighing 200–300 g were randomly assigned to three groups named the healthy control group (HC), positive control group (PC) and *L. reuteri* 17938-pretreated group (17938). All rats were housed at room temperature (22 ± 2 °C) with free access to water and standard chow. All animal experiments were performed according to the 2011 National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University. The 17938 group was gavaged daily with 1 mL of freshly

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https://doi.org/10.1016/j.biopha.2020.111000
Received 1 September 2020; Received in revised form 5 November 2020; Accepted 8 November 2020
Available online 14 November 2020

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prepared *L. reuteri* 17938 suspensions while the HC and PC groups were gavaged daily with 1 mL of sterile normal saline for 7 days. D-galactosamine (Sigma, Saint Louis, MO, USA) was intraperitoneally injected in both the PC group and the 17938 group at a dose of 1.1 g/kg body weight to induce acute liver injury. Meanwhile, the HC group received the same dose of sterile normal saline through intraperitoneal injection. Twenty-four hours after the injection of D-galactosamine, the rats were anesthetized with 400 mg/kg chloral hydrate (Sigma, Saint Louis, MO, USA). Blood, liver, terminal ileum and fecal samples from the rats were collected for further analysis.

2.3. Liver function analysis

The blood was centrifuged at 3000×g for 10 min to obtain serum, which was collected from the inferior vena cava. Then, 300 μL of serum was analyzed by biochemical analyzer (Hitachi 7600–210; Tokyo, Japan) to detect the concentration of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein, globulin, albumin, total bile acid (TBA), total bilirubin (TB), direct bilirubin (DB), indirect bilirubin (IB), gamma-glutamyl transferase (GGT) and glycylproline dipeptidyl aminopeptidase (GPDA).

2.4. Serum cytokine analysis

A Bio-Plex Pro Rat Cytokine 23-Plex Panel Assay (Bio-Rad, Hercules, California, USA) was used to determine the levels of serum cytokines, including interleukin-1α (IL-1α), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-17A, IL-18, granulocyte colony-stimulating factor (G- CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), MIP-3α, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), vascular endothelial growth factor (VEGF), growth-regulated α protein (GRO/KC) and regulated upon activation normal T-cell expressed and secreted protein (RANTES). *2.5. Histological examination*

Liver or terminal ileum tissues (0.5 × 0.5 × 0.5 cm) taken from anesthetized rats were fixed with 10 % formaldehyde solution for 24 h, and they then underwent dehydration, paraffin embedding, sectioning with a length of 2 μm, and staining with hematoxylin and eosin(HE).
Afterward, the slides were scanned by a microscope, and tissue damage was assessed. The liver biopsies were graded according to the histological activity index in four categories: periportal necrosis, intralobular necrosis, portal inflammation, and fibrosis. The combined score of the four categories formed the HAI score for each biopsy specimen [18]. Intestinal mucosal injury was graded from 0 to 5 as described by Chiu [19].

2.6. 16S rRNA gene sequencing and data analysis

DNA was extracted from rat feces using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Valencia, USA). PCR amplification of the V3–V4 regions of the bacterial 16S rRNA gene was conducted using Phusion Hot Start Flex 2X Master Mix, and the primers were 338 F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT- 3′). These amplicons were purified with AMPure XP beads (Beckman Coulter Genomics, Danvers, MA, USA) and sequenced by an Illumina MiSeq platform (Illumina, San Diego, CA, USA). Paired-end reads were merged by FLASH and then filtered according to fqtrim (v0.94) to obtain high-quality clean tags. Vsearch software (v2.3.4) was used to filter chimeric sequences and cluster similar sequences (≥97 % similarity) into the same operational taxonomic units (OTUs). Representative sequences were chosen for each OTU, and taxonomic data were then assigned to each representative sequence using the Ribosomal Database Project (RDP) classifier. Alpha diversity and beta diversity were calculated using QIIME software (v 1.8.0). *2.7. Detection and analysis of metabolites in feces*

Every 20 mg of feces was homogenized in 800 mL of ice-cold methanol (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 14,000 rpm for 15 min. The supernatant was filtered through a 0.22-μm membrane. After 20 μL of heptadecanoic acid (1 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) was added, the filtrate was concentrated to dryness under nitrogen at room temperature. The dry product was dissolved in 50 μL of methoxylamine pyridine (14 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37 °C for 24 h after 1 min of vortexing. After 50 μL of N,O-bistrifluoroacetamide containing 1% trimethylsilyl chloride was added, the mixture was derivatized at 70 °C for 2 h, and then gas chromatography (GC) analysis was performed on an Agilent 7890A- 5975C GC–MS system (Agilent, USA). Metabolites were identified by the NIST 17 databases (matching score ≥ 80 %). Orthogonal partial least squares-discriminant analysis (OPLS-DA) was performed using SIMCA software (v 14.1). *2.8. Transcriptome analysis*

Total RNA was extracted from tissues using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). RNA purity was checked using a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA), and RNA integrity was assessed using an RNA Nano 6000 Assay Kit for the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Sequencing libraries were generated using an NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using a TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina NovaSeq platform, and 150 bp paired-end reads were generated. Clean data (clean reads) were obtained by removing reads containing adapters, reads containing poly-N and low-quality reads from raw data. The index of the reference genome was built using Hisat2 v2.0.5, and paired-end clean reads were aligned to the reference genome using Hisat2 v2.0.5. FeatureCounts v1.5.0-

p3 was used to count the number of reads mapped to each gene. Then, the FPKM of each gene was calculated based on the length of the gene and read counts mapped to that gene. A differential expression analysis between two groups was performed using the DESeq2 R package (v1.16.1). We used the clusterProfiler R package to test the statistical enrichment of differentially expressed genes in KEGG pathways.

2.9. Validation of the effects of DSM 17938 using an acetaminophen- induced liver failure model

To further verify the effect of DSM 17938, we established a rat model of acute liver injury induced by acetaminophen. Eighteen male specific pathogen-free (SPF) Sprague-Dawley rats (Shanghai SLAC Laboratory Animal Co., Ltd., China) weighing 200–300 g were randomly assigned to three groups named the healthy control group (HC), positive control group (APAP) and *L. reuteri* 17938-pretreated group (APAP + 17938). The APAP + 17938 group was gavaged daily with 1 mL of freshly prepared *L. reuteri* 17938 suspensions while the HC and APAP groups were gavaged daily with 1 mL of sterile normal saline for 7 days.

Acetaminophen (Aladdin, Shanghai, China) was administered orally in both the APAP group and the APAP + 17938 group at a dose of 2 g/kg body weight to induce acute liver injury. Meanwhile, the HC group received the same dose of 0.5 % sodium carboxy methylcellulose (CMC- Na). Twenty-four hours after the administration of acetaminophen, the rats were anesthetized with 400 mg/kg chloral hydrate (Sigma, Saint Louis, MO, USA). Blood and liver samples of rats were collected for the analysis of liver function, serum cytokine levels and liver histology.

2.10. Statistical analysis

The data on liver function, serum cytokine concentration, transcriptome, liver and ileum pathological scores, gut microbiota diversity and fecal metabolic differences were analyzed by the Kolmogorov- Smirnov method. One-way ANOVA followed by the Student-Newman- Keuls method was used if data followed normal distribution characteristics; otherwise, the Mann-Whitney *U* test was used. The Wilcoxon rank sum test combined with the Benjamini-Hochberg method was applied to

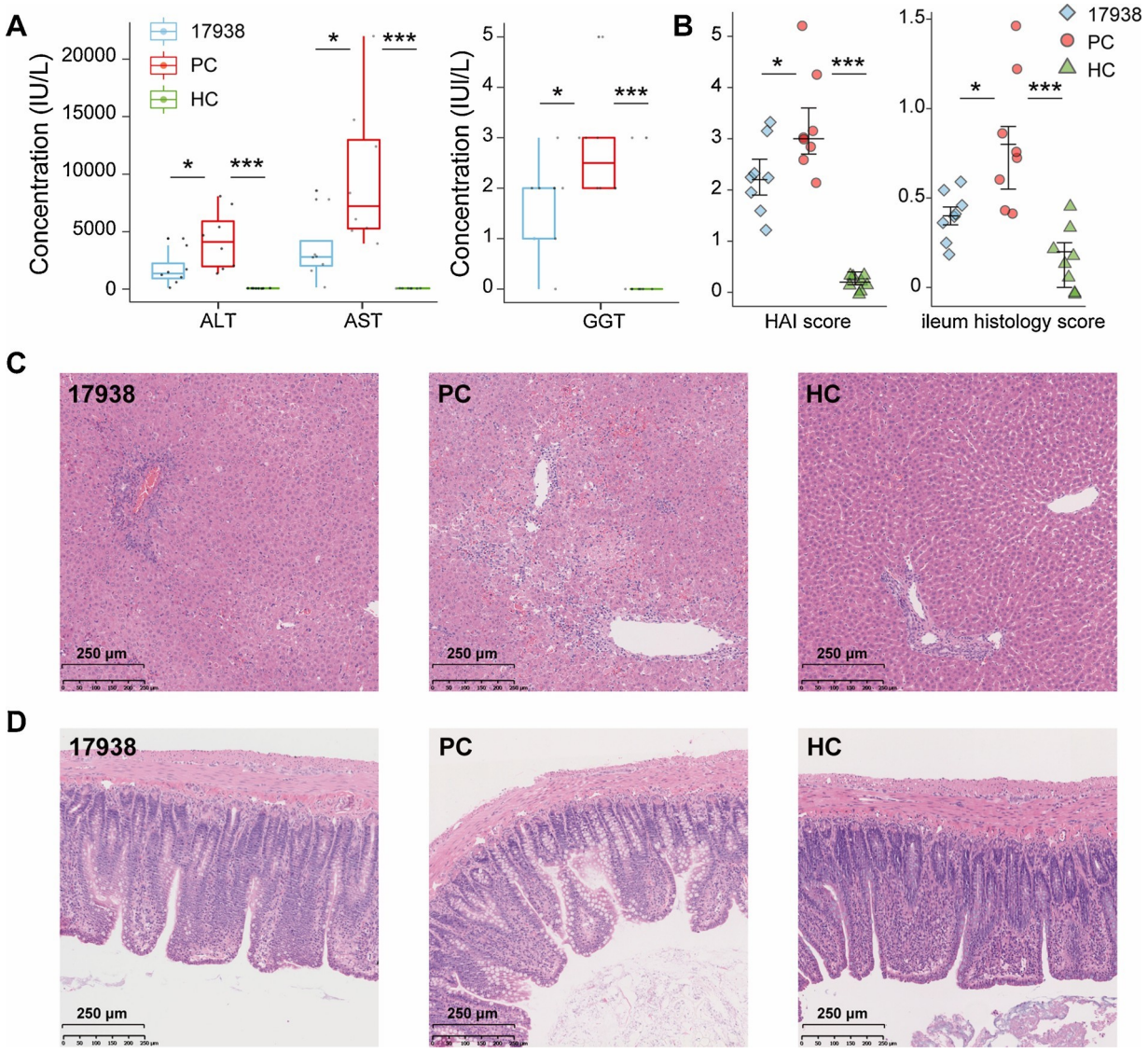

Fig. 1. *L. reuteri* DSM 17938 alleviates d-galactosamine-induced liver injury and intestinal damage. (A) Concentrations of ALT, AST and GGT in serum samples. (B) The HAI score and ileum histological score were graded based on eight samples in each group. (C) Representative images of liver and terminal ileum samples stained by H&E; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the PC group. Abbreviations: 17938, the group receiving intragastrically administered DSM 17938 and intraperitoneally injected with D-galactosamine; PC, the group receiving intragastrically administered saline and intraperitoneally injected with D-galactosamine; HC, the group receiving intragastrically administered saline and intraperitoneally injected with saline; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; HAI, histological activity index; H&E, hematoxylin and eosin.

compare the relative abundance of gut bacteria in different groups. A correlation analysis of variants was conducted using Spearman's rank correlation test. All data are presented as the mean ± SEM (standard error of the mean). Statistical significance was defined as two-sided at $p < 0.05$.

3. Results

3.1. L. reuteri DSM 17938 alleviates D-galactosamine-induced liver injury in rats

From the HC group to the PC group, the levels of serum total protein, globulin and albumin decreased while those of ALT, AST, ALP, TBA, TB, DB, IB, GGT and GPDA increased significantly. Pretreatment with *L. reuteri* DSM 17938 alleviated liver injury by reducing the increase in ALT, AST and GGT levels induced by D-galactosamine (Fig. 1A). HE staining revealed that pretreatment with *L. reuteri* DSM 17938 also alleviated hepatocyte necrosis, eosinophil colony formation and infiltration of inflammatory cells such as monocytes and lymphocytes caused by D-galactosamine (Fig. 1C). The HAI score of rats in the 17938 group was lower than that in the PC group, indicating the alleviation function of *L. reuteri* DSM 17938 on liver injury caused by D-galactosamine (Fig. 1B).

3.2. L. reuteri DSM 17938 reduces D-galactosamine-related damage to intestinal tight junctions

The results of HE staining revealed that the epithelium villi and crypts of the terminal ileum in the HC group were tightly and neatly arranged while the terminal ileum of the PC group was damaged by intraperitoneal injection of D-galactosamine. Villous rupture, denuded villi, crypt disintegration, mucosal perforation and crypt loss were observed in the PC group. *L. reuteri* DSM 17938 pretreatment ameliorated these histological abnormalities and significantly lowered ileum histological scores, indicating that pretreatment with *L. reuteri* DSM 17938 enhanced intestinal tight junctions (Fig. 1B and 1C).

3.3. L. reuteri DSM 17938 alleviates D-galactosamine-induced serum cytokine storms

To explore the effects of *L. reuteri* DSM 17938 on the immune systems during liver injury, we assayed the serum concentrations of 23 inflammation-related cytokines. Compared with those in the HC group, D-galactosamine induced increases in the levels of 15 cytokines in the PC group, including IL-1α, IL-1β, IL-2, IL-6, IL-7, IL-10, IL-13, IL-17, IL-18, GM-CSF, IFN-γ, M-CSF, MIP-1α, MIP-3α, and TNF-α, as well as a decrease in RANTES (Fig. 2). Pretreatment with *L. reuteri* DSM 17938 significantly reduced the D-galactosamine-induced increases in IL-1a, IL- 2, IL-18, M-CSF, and MIP-3α levels. Meanwhile, the concentrations of IL- 1β, IL-6, IL-7, IL-10, IL-13, IL-17, GM-CSF and IFN-γ were not significantly different between the HC group and the 17938 group, implying that DSM 17938 relieved D-galactosamine-induced alterations in the levels of cytokines.

3.4. L. reuteri DSM 17938 protects against D-galactosamine-induced beneficial bacterium depletion and pathogen enrichment

Probiotics can interact with the host through the gut microbiota. Therefore, we analyzed the effect of *L. reuteri* DSM 17938 on D-galactosamine-induced alterations in gut microbiota. We obtained 1,894,885 reads from 24 rat feces samples by 16S rRNA gene sequencing. The diversities, as estimated by the Shannon index, were not significantly different among the three groups. The species richness, as indicated by the Chao1 index, was similar between the PC group and the HC group; however, the species richness of the 17938 group was lower than that of the other two groups (Fig. 3A). According to the principal coordinate analysis (PCoA) results, the microbiota profiles of the three groups could be clearly separated, indicating that their compositions were

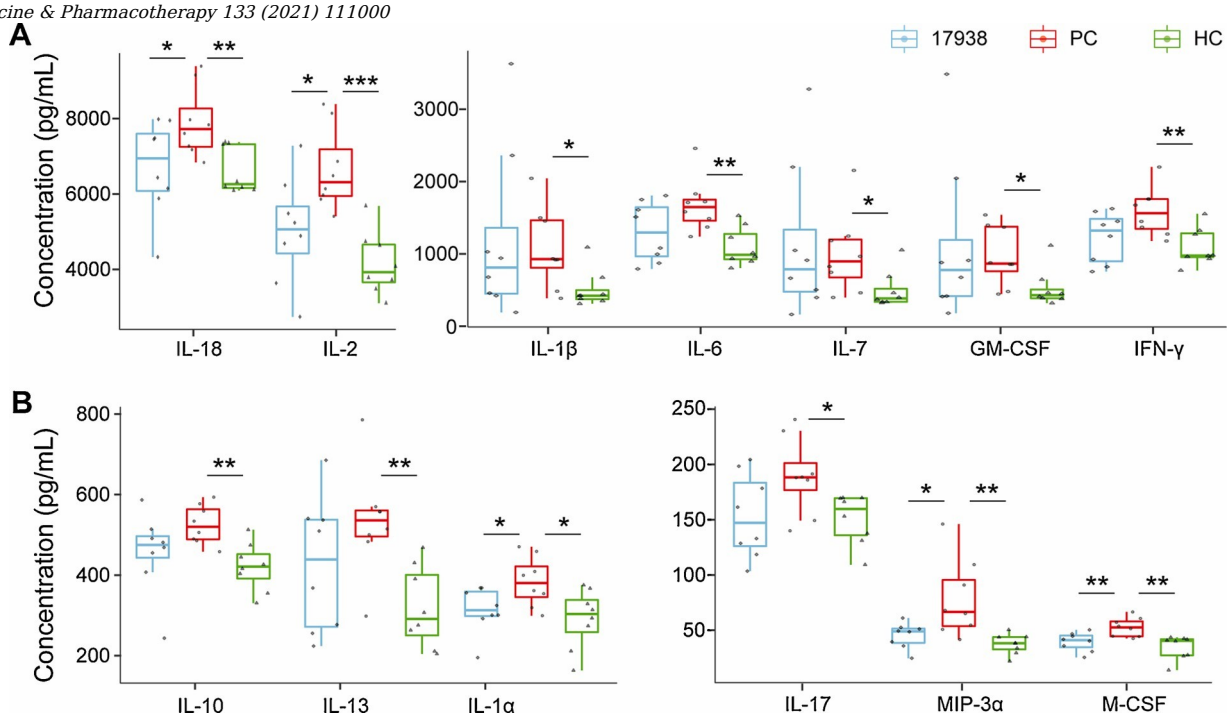

Fig. 2. *L. reuteri* DSM 17938 alleviates d-galactosamine-induced serum cytokine storms. Concentration of altered cytokines in serum samples; *p < 0.05, **p < 0.01 and ***p < 0.001 compared with the PC group. Abbreviations: 17938, the group receiving intragastrically administered DSM 17938 and intraperitoneally injected with D-galactosamine; PC, the group receiving intragastrically administered saline and intraperitoneally injected with D-galactosamine; HC, the group receiving intragastrically administered saline and intraperitoneally injected with saline; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; MIP-3α, macrophage inflammatory protein 3α; M-CSF, macrophage colony-stimulating factor.

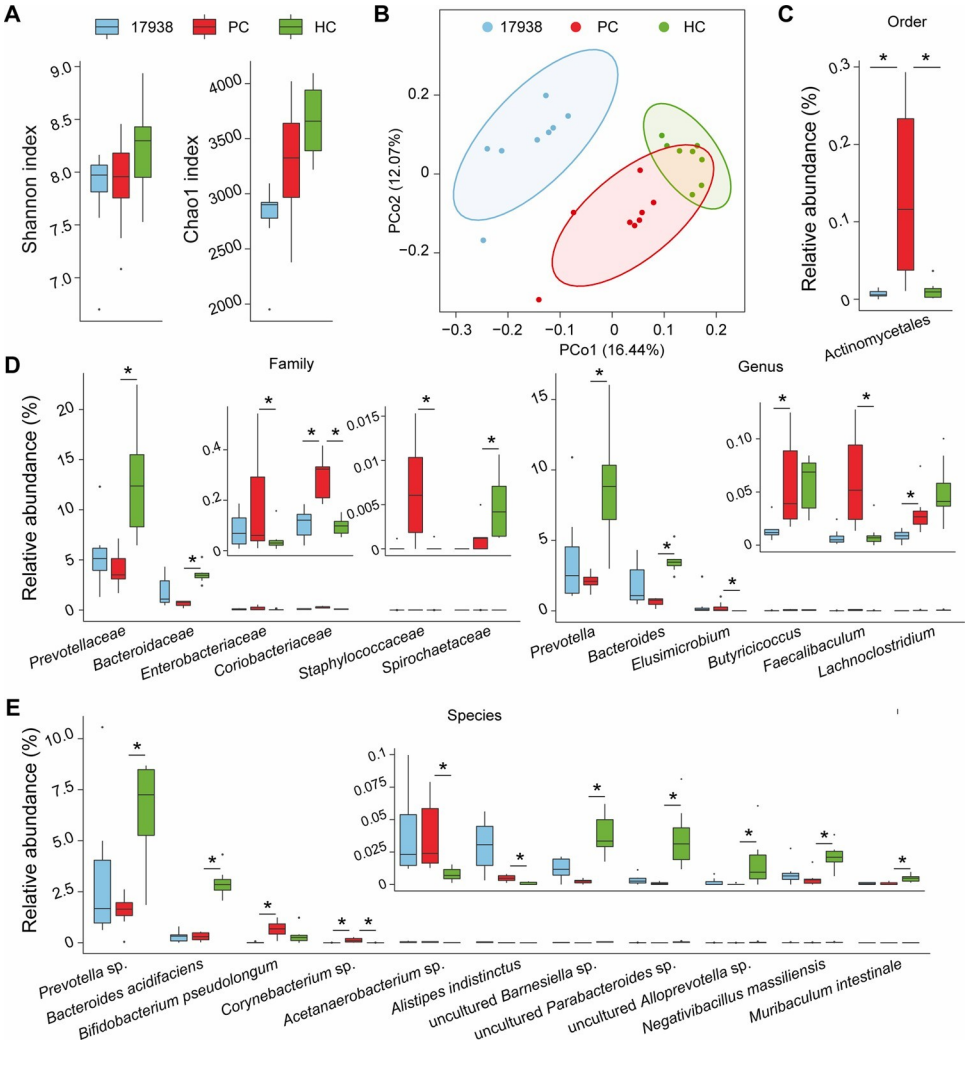

Fig. 3. *L. reuteri* DSM 17938 protects against D-galactosamine-induced beneficial bacterium depletion and pathogen enrichment. (A) Chao1 index and Shannon index describing the alpha diversity of the microbiota in the three groups. (B) Beta diversity was determined by the unweighted UniFrac distance method and PCoA. (C, D and E) Alterations in the relative abundances of bacterial taxa at the order, family, genus and species levels between the 17938, PC and HC groups; * q < 0.05, ** q < 0.01 and *** q < 0.001 compared with the PC group. Abbreviations: 17938, the group receiving intragastrically administered DSM 17938 and intraperitoneally injected with D-galactosamine; PC, the group receiving

intragastrically administered saline and intraperitoneally injected with D-galactosamine; HC, the group receiving intragastrically administered saline and intraperitoneally injected with saline.

significantly different (Fig. 3B). The results of the permutational multivariate analysis of variance also showed significant differences in the microbiota composition of these three groups ($p = 0.001$).

Compared with the HC group, the PC group was depleted of some taxa of Bacteroidales but enriched with some potentially pathogenic taxa of Actinobacteria or Firmicutes. The PC-depleted taxa of Bacteroidales included not only *Bacteroidaceae* and its members, such as *Bacteroides* and *Bacteroides acidifaciens*, but also *Prevotellaceae* and its members, such as *Prevotella* and *Prevotella* sp., as well as *Muribaculum intestinale*. *Alistipes indistinctus* was the only PC-enriched taxa belonging to Bacteroidales. In contrast, several taxa were enriched in PC feces. For example, five taxa of Actinobacteria were enriched in the PC group, including Actinomycetales, *Actinomycetales* sp., *Corynebacterium* sp., *Coriobacteriaceae*, and *Coriobacteriaceae* sp.. Similarly, several taxa of Firmicutes, such as *Staphylococcaceae* and *Enterococcaceae*, were enriched as well (Fig. 3C-E).

Pretreatment with *L. reuteri* DSM 17938 alleviated more than half of the D-galactosamine-induced alterations in the gut microbiota. First, pretreatment with DSM 17938 prevented the D-galactosamine-induced enrichment of Actinomycetales, *Actinomycetales* sp., *Coriobacteriaceae*, *Coriobacteriaceae* sp., and *Corynebacterium* sp. belonging to Actinobacteria as well as *Faecalibaculum rodentium* belonging to Firmicutes, making their relative abundances in the 17938 group close to or even lower than those in the HC group. Second, the depletion of *Bacteroidaceae*, *Bacteroides*, *Alloprevotella*, *Prevotella* sp., and *Negativibacillus massiliensis* and the enrichment of the opportunistic pathogens *Staphylococcaceae* and *Enterococcaceae* in the comparison of the PC and HC groups were not observed in the comparison of the 1798 and HC groups. This result indirectly indicates that pretreatment with DSM 17938 relieved D-galactosamine-induced alterations in the abundances of the above bacteria. In addition, several taxa, such as *Butyricicoccus*, *Lachnoclostridium* and *Bifidobacterium pseudolongum*, were depleted in the 17938 group versus in the PC group.

3.5. L. reuteri DSM 17938 slightly improves D-galactosamine-induced gut metabolic disorders

Metabolites are important links between the microbiota and the host. Therefore, we analyzed the effect of *L. reuteri* DSM 17938 on D-galactosamine-induced gut metabolic alterations. A total of 81 metabolites were identified by GC–MS. The results of the OPLS-DA analysis showed that the metabolome profiles of the HC group, the PC group and the 17938 group were separately clustered from each other, but the latter two were relatively closer (Fig. 4A). Metabolites that significantly contributed to the separation of the three metabolome profiles were selected based on variable importance for projection (VIP) values higher than 1.5 and included hexanoic acid, trisaminol, 1-hexadecanol, campesterol, D-lactose and lithocholic acid (Fig. 4B).

Subsequently, the metabolite-level differences between groups were analyzed using parametric or nonparametric tests. Seven amino acids (such as L-tyrosine and L-lysine), 4 oligosaccharides (such as L-rhamnose, maltose, and D-fructose), 4 fatty acids and their derivatives (such as

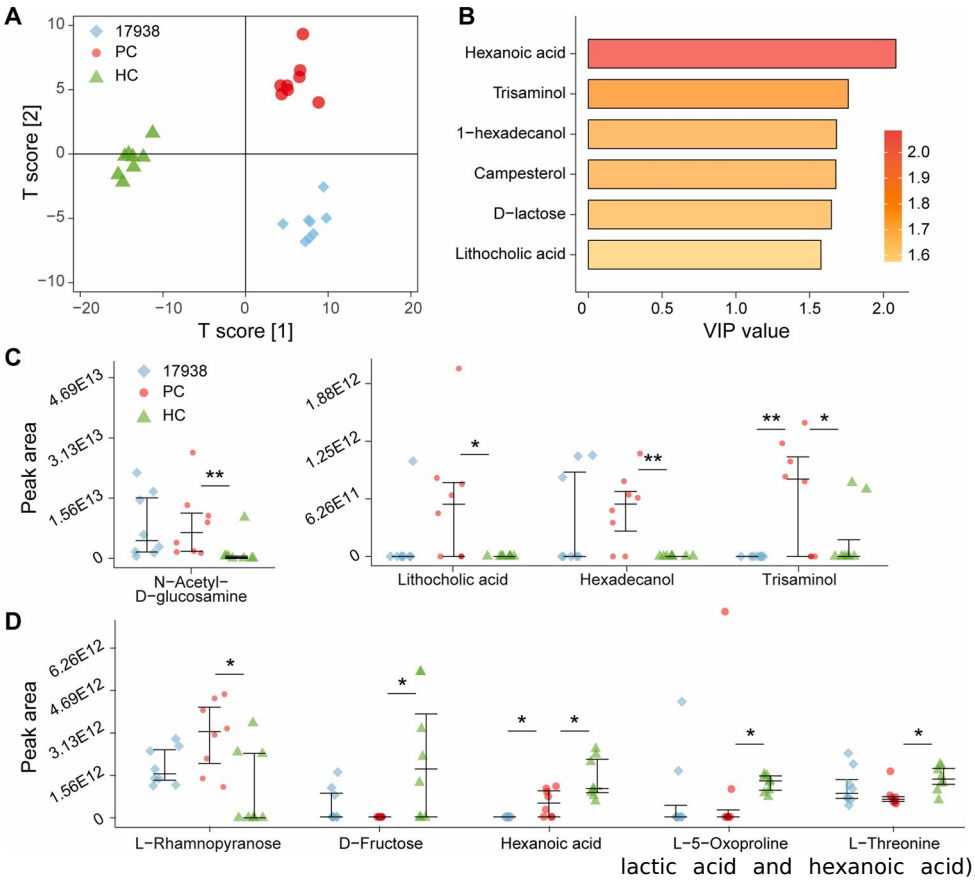

Fig. 4. *L. reuteri* DSM 17938 slightly improves d-galactosamine-induced gut metabolic disorders. (A) OPLS-DA score plots of the metabolome profiles of the 17,938, PC and HC groups detected by GC–MS analysis. (B) Metabolites selected based on VIP values higher than 1.5. (C) Peak areas in GC–MS analysis of significantly different metabolites among the
17938, PC and HC groups; *p < 0.05, **p < 0.01 and ***p < 0.001 compared with the PC group. Abbreviations: 17938, the group receiving intragastrically administered DSM 17938 and intraperitoneally injected with D-galactosamine; PC, the group receiving intragastrically administered saline and intraperitoneally injected with D-galactosamine; HC, the group receiving intragastrically administered saline and intraperitoneally injected with saline.

lactic acid and hexanoic acid), pantothenic acid, uracil and pyrimidine were depleted while 3 bile acids (deoxycholic acid, lithocholic acid and chenodeoxycholic acid), 3 alcohols (1-hexadecanol, cholestan-3-ol and trisaminol), phosphoric acid and N-acetyl-D-glucosamine were enriched in the feces of the PC group versus those in the feces of the HC group. These results indicate that D-galactosamine impaired gut metabolism in rats. Furthermore, the PC-enriched deoxycholic acid, lithocholic acid, and chenodeoxycholic acid suggest that the enterohepatic circulation of bile acids was disrupted by D-galactosamine.

Pretreatment with DSM 17938 reduced the D-galactosamine-induced enrichment of trisaminol and further reduced the D-galactosamine- induced decrease in hexanoic acid. Furthermore, after pretreatment with DSM 17938, D-galactosamine did not induce the significant alterations in the levels of 1-hexadecanol, D-fructose, L-rhamnopyranose, L-5-oxoproline, L-threonine, lithocholic acid, or N-acetyl glucosamine methoxime in rat feces as it did in the PC group (Fig. 4C). These results indicate that DSM 17938 alleviated gut metabolic disorders during D-galactosamine-induced liver injury.

3.6. L. reuteri DSM 17938 reduces D-galactosamine-induced upregulation of inflammatory gene transcripts in the liver

In transcriptome analysis, transcript abundances of 339 genes were altered in not only the comparison of the HC and PC groups but also that of the DSM 17938 and PC groups. Taking the ratio of transcript abundance in HC to that in PC and/or the ratio of DSM 17938 to PC more than 16 or less than

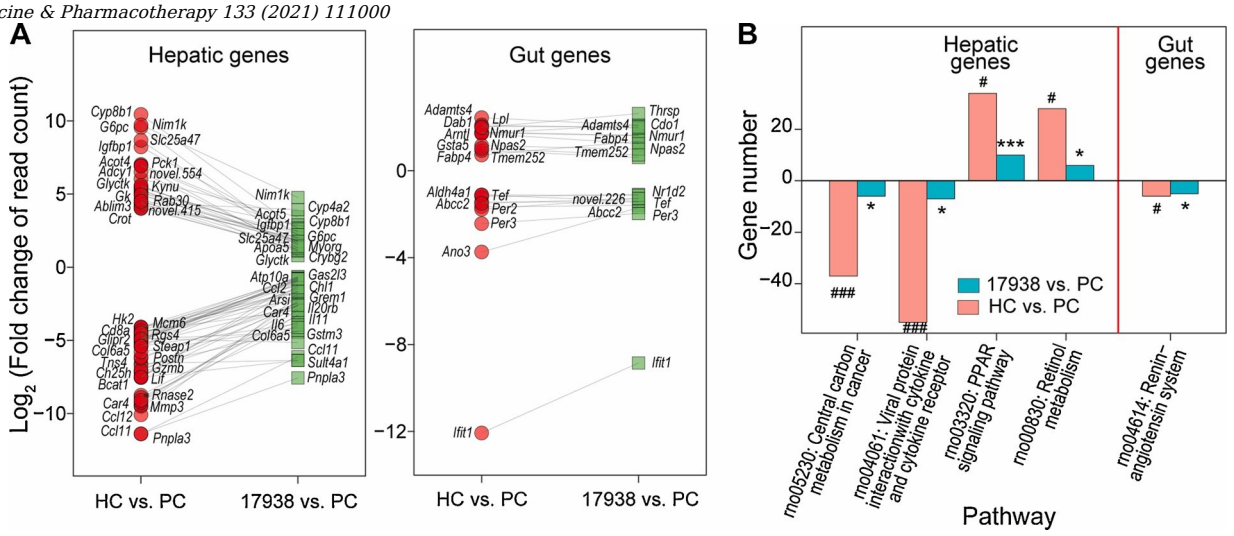

Fig. 5. *L. reuteri* DSM 17938 attenuates D-galactosamine-induced alterations in some gene transcripts and several pathways in the liver and gut. (A) *L. reuteri* DSM 17938 attenuates not only the D-galactosamine-induced upregulation of 46 genes and downregulation of 28 genes in the liver but also the D-galactosamine-induced upregulation of 10 genes and downregulation of 12 genes in the gut. (B) KEGG enrichment analysis of hepatic and gut genes the differential transcription of which was caused by D-galactosamine or *L.reuteri* DSM 17938 administration. The number of upregulated genes is shown above the zero axis, and the number of downregulated genes is shown below the zero axis. Enrichment significance in 17938 versus PC: * P_{adj} <0.05, ** P_{adj} < 0.01, *** P_{adj} < 0.001; enrichment significance in HC versus PC: # P_{adj} < 0.05, ## P_{adj} < 0.01, ### P_{adj} < 0.001. Abbreviations: 17938, the group receiving intragastrically administered DSM 17938 and intraperitoneally injected with D-galactosamine; PC, the group receiving intragastrically administered saline and intraperitoneally injected with D-galactosamine; HC, the group receiving intragastrically administered saline and intraperitoneally injected with saline.

1/16 as the threshold (log_2 (fold change of read count) >4 or < -4), 74 genes were selected and discussed. First, pretreatment with DSM 17938 significantly reduced the D-galactosamine-induced dramatic transcriptional upregulation of 46 genes, especially those of many inflammation-related genes, such as four chemokine genes, *Ccl2*, *Ccl7*, *Ccl11* and *Ccl12*; three interleukin genes, *Il6*, *Il11* and *Il20rb*; two matrix metalloprotease genes, *Mmp3* and *Mmp10*; and the tumor necrosis factor-related gene *Tnfaip6*. Second, pretreatment with DSM 17938 partially reversed the D-galactosamine-induced tremendous transcriptional downregulation of 28 genes in rat liver. Some of them are critical to life activities and drug metabolism, such as the cytochrome P450 genes *Cyp4a2* and *Cyp8b1* and the acyl− COA thioesterase genes *Acot4* and *Acot5*. In addition, DSM 17938 pretreatment exacerbated the D-galactosamine-induced transcriptional upregulation of the *Ret* and *Ccr7* genes (Fig. 5A). As shown from the pathways reconstructed in KEGG, DSM 17938 pretreatment alleviated not only D-galactosamine-induced downregulation of retinol metabolism, and the PPAR signaling pathway but also the upregulation of viral protein interaction with cytokine and cytokine receptor and central carbon metabolism in cancer signaling pathway (Fig. 5B).

3.7. L. reuteri DSM 17938 attenuates D-galactosamine-induced alterations of some gene transcripts in the gut

In the transcriptome analysis, the transcript abundances of 22 intestinal genes were significantly different not only between the HC and PC groups but also between the 17938 and PC groups. First, pretreatment with DSM 17938 reduced the D-galactosamine-induced increase in transcripts of 10 genes, such as *Ifit1*, *Abcc2*, *Aldh4a1*, *Ano3*, *Nr1d2*, *Per2*, *Per3*, *Pparg*, and *Tef*. Strikingly, the transcript count of *Ifit1* in the PC group was nearly 4200 times greater than that in the HC group; however, its count in the 17938 group was only 10 times greater than that in the HC group. Second, pretreatment with DSM 17938 significantly restored the D-galactosamine-induced transcriptional downregulation of 12 genes, *Fabp4*, *Adamts4*, *Arntl*, *Ccn1*, *Cdo1*, *Dab1*, *Gsta5*, *Lpl*, *Nmur1*, *Npas2*, *Thrsp* and *Tmem252* (Fig. 5A). As shown from the pathways reconstructed in KEGG, the KEGG pathway enrichment analysis revealed that L. *reuteri* DSM 17938 pretreatment significantly alleviated D-galactosamine-induced downregulation of the renin-angiotensin system signaling pathway (Fig. 5B).

3.8. Correlations between the gut microbiota, fecal metabolome, immunity, liver function and transcriptome

A direct interaction exists between gut microbes and gut metabolites; thus, we first analyzed the correlation between them; and p < 0.001 was used as a screening threshold for finding major correlations. In Fig. 6A, campesterol was correlated with all of the shown bacterial taxa except for *Staphylococcaceae* and Actinomycetales; these correlations were mainly positive with members of Bacteroidetes, such as *Bacteroides acidifacions*, *Muribaculum intestinale*, and *Barnesiella* sp.. In addition, campesterol was negatively correlated with *Alistipes indistinctus* and *Acetanaerobacterium* sp.. Moreover, trisaminol was positively correlated with *Staphylococcaceae* and Actinomycetales, which contain many opportunistic pathogens. N-acetyl-D-glucosamine was negatively correlated with *Prevotellaceae* (Fig. 6A).

Most detected members of Bacteroidetes, campesterol and D-galactose were negatively correlated with inflammatory factors but positively correlated with transcripts of genes important to life. First, serum AST and AST were negatively correlated

with *Prevotellaceae*, *Bacteroides*, *Muribaculum intestinale*, *Alloprevotella sp.*, *Barnesiella* sp., and *Parabacteroides* sp., among which *Prevotellaceae* was also negatively correlated with serum IL-10, IL-2 and MIP-3α (Fig. 6B). At the transcript level, *Barnesiella* sp. was widely negatively correlated with inflammation- related liver genes such as *Il11*, *Lif*, *Mmp3* and *Cd8a*, and other correlations included *Prevotellaceae* with *Rgs4*, *Parabacteroides* sp.with *Lif* and *Mmp3*, and *Prevotellaceae* or *Barnesiella* sp. with *Ano3* (Fig. 6C and D). For gut metabolites, negative correlations were found for campesterol with serum AST and AST and for D-galactose with serum GM-CSF, IL-1β and IL-7 (Fig. 6B). Second, gut *Prevotellaceae* was positively correlated with transcripts of not only liver *Cyp8b1*, *G6pc*, *Acot4* and *Pex11a* but also gut *Gsta5*, *Adamts4* and *Tmem252*, which are important to life activities (Fig. 6C and D).

Some detected bacterial products and taxa rich in opportunistic pathogens were positively correlated with inflammatory-related factors but negatively correlated with factors important to life. Serum AST and ALT were positively correlated with gut *Corynebacterium* sp., *Elusimicrobium* and N-acetyl-D-glucosamine, and *Staphylococcaceae* was positively correlated with MIP-3α (Fig. 6B). Interestingly, the transcripts of the cell adhesion molecule L1-like gene (*Chl1*) in the liver were highly positively correlated with gut *Staphylococcaceae*, *Corynebacterium* sp., and trisaminol; furthermore, *Coriobacteriaceae* was negatively correlated with transcripts of genes such as *Cdo1*, *Fabp4*, *Lpl* and *Thrsp* related to the metabolism of fatty acids but positively correlated with *Pparg*, *Nr1d2*, and *Aldh4a1* (Fig. 6C and D).

Three gut genes, *Nmur1*, *Ano3* and *Tmem252*, were widely associated with liver genes at the transcript level. Gut *Nmur1* was positively correlated with liver *Abcg5*, *Ablim3*, *Acot4*, *Acot5*, *Crot*, *Crybg2*, *Cyp8b1*, *Gk*, *Glyctk*, *Kynu*, *Myorg*, *Pck1* and *Pex11a*. Gut *Ano3* was negatively correlated with gut *Crot*, *Cyp8b1*, *Myorg*, *Pex11a*, and *Rab30* but positively correlated with *Rgs4*. Gut *Tmem252* was positively correlated with liver *Acot5*, *Atf5*, *Cyp8b1*, *G6pc* and *Rab30*(Fig. 6E).

3.9. DSM 17938 can also alleviate acetaminophen-induced acute liver failure

The effect of DMS 17938 on acute liver failure was further verified using a rat model of acetaminophen hepatotoxicity. Histological examination showed that 17938 pretreatment significantly reduced liver hemorrhage, hepatocyte necrosis and inflammatory cell infiltration caused by acetaminophen and reduced the overall pathological score (Fig. 7A). Furthermore, pretreatment with 17938 significantly reduced the acetaminophen-induced increase in serum ALT and AST (Fig. 7C). Meanwhile, compared with that in the HC group, the serum level of GGT was significantly increased in the acetaminophen group, but it was not significantly altered in the group treated with both 17938 and acetaminophen (Fig. 7C); a similar trend was observed with MCP-1 and MIP- 3α (Fig. 7B). These results are consistent with the main preventive and therapeutic effects of 17938 on D-galactose-induced liver injury mentioned above.

4. Discussion

The effects of probiotics on liver diseases have received much attention. *Lactobacillus reuteri* DSM 17938 has been widely used as a drug in the treatment of diarrhea and other diseases in many countries. However, the application of DSM 17938 in acute liver injury has not

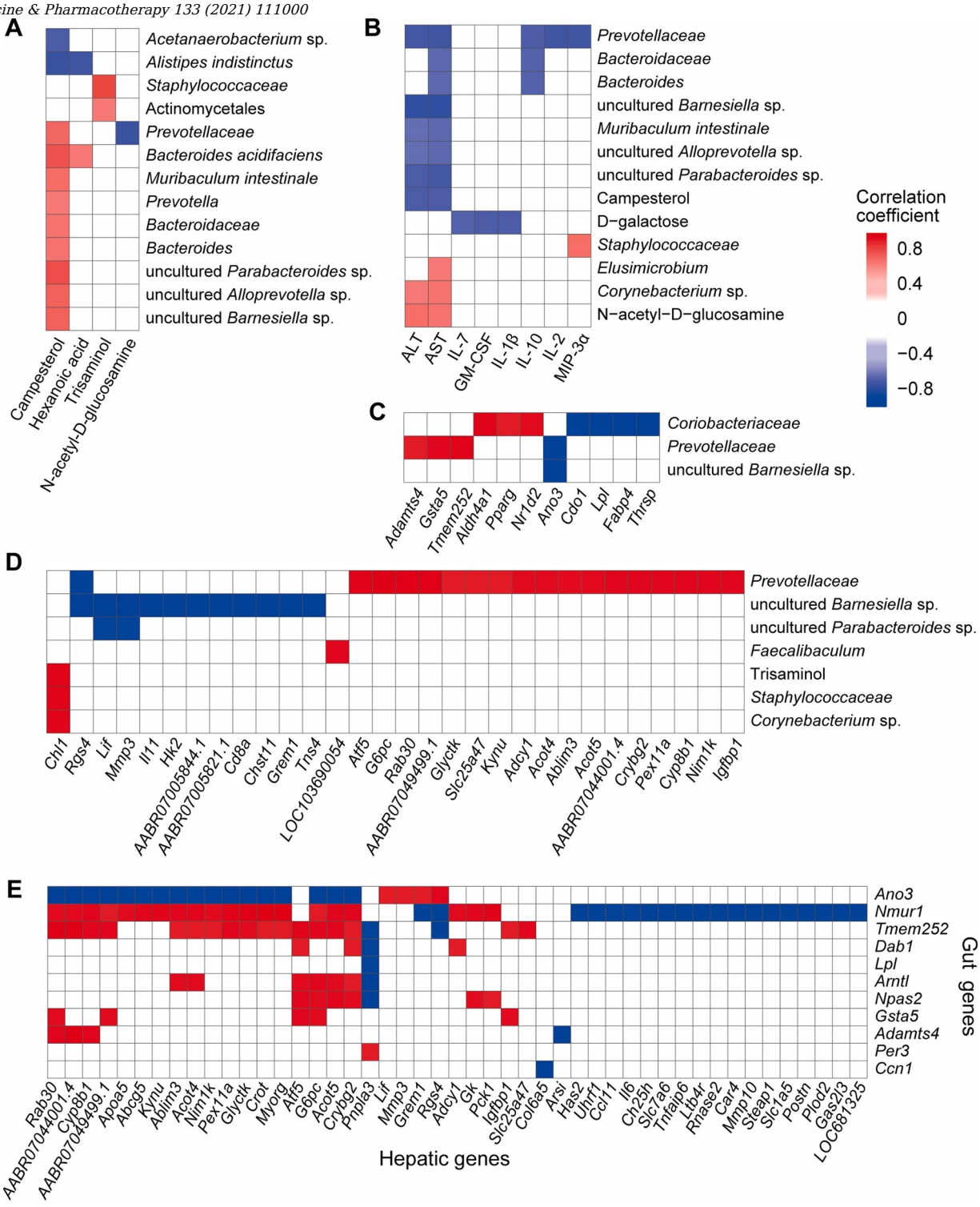

Fig. 6. Correlations between the gut microbiota, fecal metabolome, immunity, liver function and liver and intestinal transcriptomes. (A) Correlations of gut microbes with gut metabolites. (B) Correlations of these gut microbes and metabolites with liver function indicators and inflammatory cytokines. (C) Correlations of these gut microbes and metabolites with gut gene transcripts. (D) Correlations of these gut microbes and metabolites with hepatic gene transcripts. (E) Correlations of hepatic gene transcripts with gut gene transcripts. These results with P values <0.001 are shown in the heatmap. The colour key indicates the strength of the correlation. Red indicates positive correlations; blue indicates negative correlations. Abbreviations: 17938, the group receiving intragastrically administered DSM 17938 and intraperitoneally injected with D-galactosamine; PC, the group receiving intragastrically administered saline and intraperitoneally injected with D-galactosamine; HC, the group receiving intragastrically administered saline and intraperitoneally injected with saline; ALT, alanine aminotransferase; AST, aspartate aminotransferase; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; MIP-3α, macrophage inflammatory protein 3α.

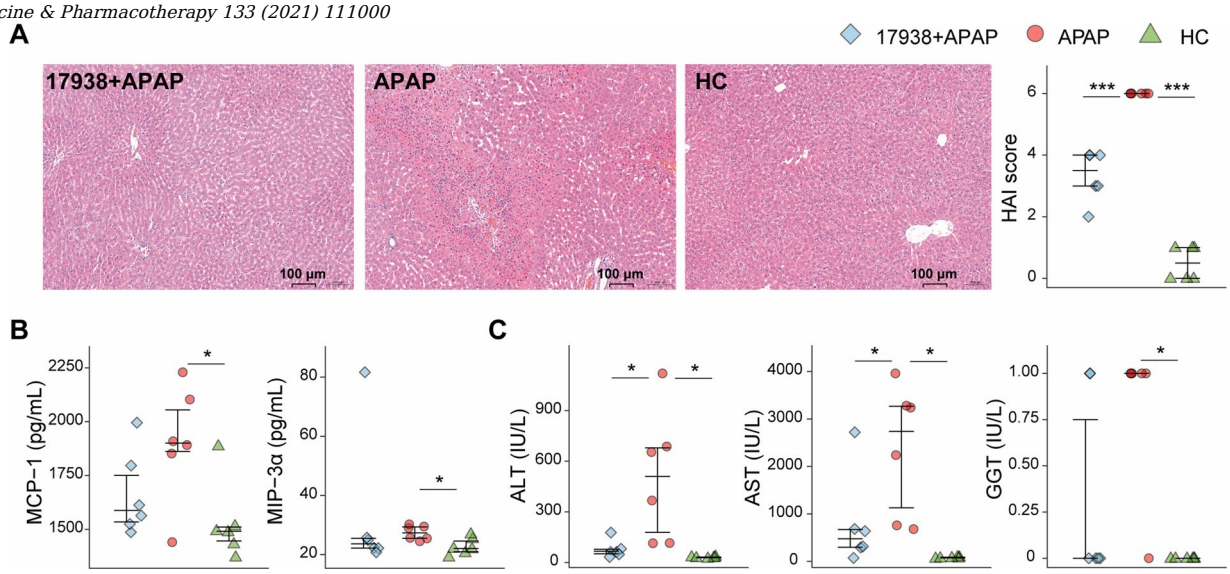

Fig. 7. *L. reuteri* DSM 17938 alleviates acetaminophen-induced acute liver failure. (A) Representative images of liver samples stained by H&E and the HAI score graded based on six samples in each group. (B) Concentration of MCP-1 and MIP-3α in serum samples. (C) Concentrations of ALT, AST and GGT in serum samples; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the PC group. Abbreviations: 17938 + APAP, the group receiving intragastrically administered DSM 17938 and acetaminophen; APAP, the group receiving intragastrically administered saline and acetaminophen; HC, the group receiving intragastrically administered saline and 0.5 % sodium carboxy methylcellulose (CMC-Na); H&E, hematoxylin and eosin; HAI, histological activity index; MCP-1, monocyte chemoattractant protein 1; MIP- 3α, macrophage inflammatory protein 3α; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase.

been reported to date. Our results showed that pretreatment with DSM 17938 alleviated D-galactosamine-induced liver injuries, such as liver histological abnormalities, an increase in serum cytokines, changes in the gut microbiota and microbiome, and alterations in the gene transcripts in the liver and gut. The underlying mechanisms include the downregulation of retinol metabolism and the PPAR signaling pathway as well as the upregulation of viral protein interaction with cytokine and cytokine receptor and central carbon metabolism in cancer signaling pathway. These results provide new insights for the prevention and treatment of acute liver injury.

The elevation of inflammatory factors is an important feature of the development of liver disease. Our results showed that pretreatment with DSM 17938 reduced the D-galactosamine-induced increase in the levels of inflammatory factors, such as the serum levels of AST, ALT, GGT, IL- 1α, IL-2, IL-18, M-CSF and MIP-3α and the transcription of *Ccl2*, *Ccl7*, *Ccl11*, *Ccl12*, *IL-6*, *IL-11*, *IL-20rb*, *Mmp3*, *Mmp10* and *Tnfaip6* in the liver. Elevated serum ALT and AST are highly sensitive markers of liver cell damage or liver inflammation. Both IL-11 and IL-6 belong to the IL-6 family. IL-11 inhibits the translocation of NF-κB and reduces the production of pro-inflammatory mediators, such as IL-6 [20]. CCL2, CCL7, CCL11 and CCL12, with CCR2 as a common receptor, can drive pro-inflammatory cells to an inflamed or injured area. IL-20 receptor-B is a receptor of IL-19, IL-20, and IL-24, which are associated with chronic inflammation [21]. MMP 3 has been linked with disruption of barriers between endothelial cells and the nervous system. MMP 10 controls the resolution phase of inflammation [22]. Tumor necrosis factor-inducible gene 6 protein has recently been shown to protect the liver from acute damage [23]. IL-1α and IL-18, belonging to the IL-1 family, are both pro-inflammatory cytokines. Therefore, *L. reuteri* DSM 17938 reduced D-galactosamine-induced liver inflammation.

The depletion of potentially beneficial bacteria, such as Bacteroidetes, is commonly observed in the gut microbiota of patients with liver diseases. Our results showed that certain members of the Bacteroidetes, such as *Prevotellaceae*, *Bacteroides*, *Bacteroides acidifaciens*, *Alloprevotella* sp., and *Muribaculum intestinale*, were negatively correlated with at least one of the following: AST, ALT, GGT, IL-1α, IL-2, IL- 18, M-CSF and MIP-3α or the liver transcripts of *Ccl2*, *Ccl7*, *Ccl11*, *Ccl12*, *IL-6*, *IL-11*, *IL-20rb*, *Mmp 3*, and *Mmp 10*. Several members of *Bacteroides*, especially *B. fragilis*, can facilitate the proliferation of T cells, restrict the hyperexpansion of invariant natural killer T cells during neonatal development, and protect the host from fetal damage by initially creating minor inflammation. *Bacteroides acidifaciens* promotes IgA production in the large intestine by inducing germinal center formation and increasing IgA–secreting B cells in germ-free mice [24]. Therefore, members of *Bacteroides* are widely considered candidate probiotics for the treatment of intestinal colitis, immune dysfunction, metabolic disorders and cancer [25]. Several strains of *Prevotellaceae*, especially those of *Prevotella*, play an important role in immune regulation although some appear to be inflammophilic pathobionts. For example, human gut-derived *Prevotella histicola* was reported to modulate the immune response and suppress experimental autoimmune encephalomyelitis, multiple sclerosis, collagen-induced arthritis, and rheumatoid arthritis [26]. Our results showed that D-galactosamine-induced depletion of members of Bacteroidetes may have an important effect on the immune system of the host. After pretreatment with DSM 17938, the D-galactosamine-induced depletions of *Bacteroidaceae*, *Bacteroides* and *Prevotella* in rats

were no longer significantly different from those of the HC group. Therefore, DSM 17938 may contribute to immune homeostasis by regulating the gut microbiota.

The enrichment of opportunistic pathogens in the gut microbiota, which seriously damages health, is another important finding in patients with liver diseases. First, our results showed that ALT, AST, GGT, IL-2, and MIP-3α were positively correlated with *Enterococcaceae* or *Staphylococcaceae* at the $p < 0.05$ level. Certain members of *Enterococcaceae* and *Staphylococcaceae* have already been reported to be involved in the development of liver disease. For example, overgrowth of intestinal *Enterococcus* induced by gastric acid suppression was reported to promote alcoholic liver disease [27]. *Staphylococcus aureus* is the most common cause of spontaneous bacterial peritonitis and bacteremia in patients with liver cirrhosis and the second cause of bacteremia in patients with end-stage liver disease or in patients waiting for liver transplantation [28]. *L. reuteri* DSM 17938 pretreatment reduced the enrichment of D-galactosamine-induced *Enterococcaceae* and *Staphylococcaceae*, which helped to alleviate associated inflammation, spontaneous peritonitis, and bacteremia. Second, the relative abundance of *Coriobacteriaceae* sp. was positively correlated with not only gut lithocholic acid and trisaminol but also ALT, AST, GGT, IL-18, IL-2, M-CSF and MIP-3α at the $p < 0.05$ level. Members of *Coriobacteriaceae* play an important role in the conversion of cholesterol-derived host metabolites as well as the activation of food polyphenols. Systemic hypercholesterolemia was detected in a gnotobiotic mouse model colonized with a consortium of four strains from *Coriobacteriaceae* regardless of the diet. However, certain members of *Coriobacteriaceae* are considered pathobionts because of their implications in the development of various clinical pathologies, including bacteremia, periodontitis and vaginosis [29]. Our results showed that *Coriobacteriaceae* and an unknown species belonging to it were significantly enriched in the PC group compared with those in the HC group. However, the distribution of these two taxa between the 17938 and HC groups was not significant, indicating that DSM 17938 can alleviate the potential pathological effects caused by elevated *Coriobacteriaceae* abundance.

The liver is an important detoxification organ for gut metabolites associated with the occurrence and development of various liver diseases. Our results demonstrated that the serum ALT, AST, GGT, IL-18, IL- 2, M-CSF and MIP-3α levels were positively correlated with at least one of the following: gut N-acetyl-D-glucosamine, 1-hexadecanol, and trisaminol. The enrichment of N-acetyl-D-glucosamine and 1-hexadecanol in the PC group may not cause further health damage as they can be used as food additives. However, trisaminol has been reported to induce not only rare cases of liver toxicity in newborn babies but also hyperkalemia and pulmonary dysfunction. Therefore, the enrichment of trisaminol can further aggravate liver injury. Pretreatment with DSM 17938 reduced the increase in trisaminol levels caused by D-galactosamine and the level of trisaminol was significantly lower in the 17938 group than in the HC group, which is likely to be an important mechanism for *L. reuteri* DSM 17938 to alleviate liver injury. Strikingly, we found that the gut metabolites were far less related to the transcripts of intestinal genes than to those of liver genes, further indicating that the intestinal cells are tolerant of intestinal metabolites and that the liver plays an important role in the detoxification of intestinal metabolites.

The signaling pathway reflects the underlying mechanism of how the gut microbiota affects the occurrence and development of liver diseases. Pretreatment with *L. reuteri* DSM 17938 alleviated the D-galactosamine- induced downregulation of retinol metabolism and the PPAR signaling pathway as well as the upregulation of viral protein interaction with cytokine and cytokine receptor and central carbon metabolism in cancer signaling pathway in the liver. Meanwhile, the upregulation of renin- angiotensin system signaling pathways induced by D-galactosamine in the intestine was reduced by DSM 17938 pretreatment. PPAR-gamma signaling has anti-inflammatory, anti-fibrogenic and anti-proliferative functions in the liver. Retinoic acid is a metabolite of vitamin A that can act synergistically with PPAR-gamma signaling in various cell types [30]. Therefore, reducing the downregulation of liver retinol metabolism and the PPAR signaling pathway might be an important mechanism for DSM 17938 to alleviate liver damage. Moreover, reducing the upregulation of viral protein interaction with cytokine and cytokine receptor and central carbon metabolism in cancer signaling pathway, which can alleviate liver inflammation and liver tumors, may be another important mechanism for the protective effect of DSM 17938 on liver damage [31]. Compared with those in the PC group, the transcripts of key genes of the renin-angiotensin system, such as *Ace* (encoding angiotensin-converting enzyme), in the intestine in the 17938 group were downregulated, thereby reducing the activity of angiotensin II and inflammation, which may be the third important mechanism of DSM 17938 against liver injury [32]. Interestingly, interplay between the renin-angiotensin system and the PPAR-γ system has been reported. Some PPAR-γ agonists inhibited both the expression of angiotensin II type 1 receptor and its mediated inflammation in vivo. In turn, angiotensin II reduces PPAR-γ mRNA expression [33]. Pretreatment with DSM 17938 alleviated D-galactosamine-induced downregulation of PPAR signaling in the liver and upregulation of the renin-angiotensin signaling in the intestine, suggesting that the interplay between these two signalings may be involved in the mechanism of DSM 17938 reducing liver injury.

Our results show that DSM 17938 can also alleviate acetaminophen- induced acute liver failure. This suggests that DSM 17938 has a wide application prospect in the prevention and treatment of acute liver failure. However, some limitations exist in this study, such as not exhausting all models of acute liver failure and needing to be further verified in patients.

5. Conclusion

Pretreatment with *L. reuteri* DSM 17938 can relieve D-galactosamine- induced liver injury in rats, alleviate the disruption of the gut microbiota and metabolome, and reduce the transcription of inflammatory factors in the liver and gut. The mechanism is considered to be related to the upregulation of liver retinol metabolism and the PPAR signaling pathway, downregulation of liver viral protein interaction with cytokine and cytokine receptor and central carbon metabolism in cancer pathway and the intestinal renin-angiotensin system pathway. Therefore, this work reveals the prospects for the application of DSM 17938 in the prevention of acute liver injury.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

This work is supported by the National Key Research and Development Program of China (No. 2018YFC2000500); the National Natural Science Foundation of China (Nos.81570512, 81790631 and 81790634); the Natural Science Foundation of Zhejiang Province in China (No.LQ19H030007); and the General scientific research projects of Zhejiang Education Department (No. Y201738071).

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